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Rapid and Simple Method for Purification of Nucleic Acids

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We have developed a simple, rapid, and reliable protocol for the small-scale purification of DNA and RNA from, e.g., human serum and urine. The method is based on the lysing and nuclease-inactivating properties of the chaotropic agent guanidinium thiocyanate together with the nucleic acid-binding properties of silica particles or diatoms in the presence of this agent. By using size-fractionated silica particles, nucleic acids (covalently closed circular, relaxed circular, and linear double-stranded DNA; single-stranded DNA; and rRNA) could be purified from 12 different specimens in less than 1 h and were recovered in the initial reaction vessel. Purified DNA (although significantly sheared) was a good substrate for restriction endonucleases and DNA ligase and was recovered with high yields (usually over 50%) from the picogram to the microgram level. Copurified rRNA was recovered almost undegraded. Substituting size-fractionated silica particles for diatoms (the fossilized cell walls of unicellular algae) allowed for the purification of microgram amounts of genomic DNA, plasmid DNA, and rRNA from cell-rich sources, as exemplified for pathogenic gram-negative bacteria. In this paper, we show representative experiments illustrating some characteristics of the procedure which may have wide application in clinical microbiology.

The use of nucleic acid (NA) probes in the detection of human pathogens has become increasingly important (for reviews, see references 18 and 24). The purification of NA from specimens like serum, urine, or bacterial cultures, however, has been laborious and time-consuming. Moreover, the many steps involved in the purification of NA from such specimens by classical procedures (involving detergent-mediated lysis, proteinase treatment, extractions with organic solvents, and ethanol precipitation) increase the risk of transmission of NA from sample to sample. When the extremely sensitive polymerase chain reaction (PCR) (21, 22) or the transcription-based amplification system (13) is used for the detection of a few NA molecules of a pathogen, the transmission of NA might easily lead to false-positive results.

The binding of DNA in the presence of the chaotropic agents NaI or $NaClO_4$ to silica or glass particles is well known (16, 26, 29) but has not, to our knowledge, resulted in methods for NA purification directly from clinical specimens like human serum or urine. The NA-binding properties of fossilized cell walls of unicellular algae (hereafter called diatoms) or silica or glass particles have not been evaluated for the chaotropes guanidinium thiocyanate (GuSCN) or guanidinium hydrochloride (GuHCl). GuSCN has been shown to be a powerful agent in the purification and detection of both DNA and RNA because of its potential to lyse cells combined with its potential to inactivate nucleases (6–8, 10, 15, 25, 28). Guanidinium hydrochloride has also been successfully used in NA purification from mammalian cells but has appeared to be less effective than GuSCN with respect to RNase inhibition (4–6, 9, 28).

In this paper, we describe a method for the rapid purification of NA (covalently closed circular, relaxed circular, and linear double-stranded DNA; single-stranded DNA; and rRNA) directly from human serum and urine.

The method is based on our observation that, in the presence of high concentrations of the chaotropic agent GuSCN, NA will bind to diatoms or silica or glass particles. The method was developed to fulfill the following criteria. First, the method should be sensitive, reproducible, rapid, and simple, requiring no specialized equipment or specialized knowledge of biochemistry, thus allowing for NA purification from a large series of clinical specimens in a routine setting. Second, extracted NA should be sufficiently pure to allow for enzymatic modifications. Third, risks for personnel with regard to pathogens should be small. Finally, the chance of transmission of NA from sample to sample should be small. Although of extreme importance for PCR, we have not yet evaluated this topic in view of other expected problems associated with PCR (see, e.g., references 14 and 23).

These demands have resulted in a highly standardized method (protocol Y/SC) for NA purification from human urine and serum which uses size-fractionated silica particles as the NA carrier. Substitution of this NA carrier by diatoms allowed for the purification (protocol Y/D) of NA from cell-rich sources, as demonstrated for several pathogenic gram-negative bacteria.

MATERIALS AND METHODS

Outline of the procedure. A small sample (e.g., of human serum or urine or bacteria scraped from plates) was pipetted into a reaction vessel containing a solid NA carrier (size-fractionated silica particles or diatoms) and a GuSCN-containing lysis buffer. Lysis of mammalian cells, viruses, and gram-negative bacteria took place, and released NA was bound to the NA carrier, forming complexes which could be rapidly sedimented by centrifugation. These complexes were then washed twice with a GuSCN-containing washing buffer, twice with 70% ethanol, and once with acetone; the complexes were dried, and NA was subsequently eluted in an aqueous low-salt buffer in the initial reaction vessel. NA could be purified from 12 samples within 1 h. The procedure is inexpensive (less than $0.50 per purification for materials), and no special laboratory equipment is needed.

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Chemicals and buffers. (i) Preparation of size-fractionated SC. Silica particles (60 g of silicon dioxide, SiO_2; Sigma Chemical Co., St. Louis, Mo.) were suspended in demineralized water in a total volume of 500 ml in a glass cylinder (height of aqueous column, 27.5 cm; width, 5 cm) and sedimented at unit gravity for 24 h at room temperature. A 430-ml portion of the supernatant was then disposed of by suction, demineralized water was added to a total volume of 500 ml, and the silica pellet was suspended by vigorous shaking. After another sedimentation step (5 h at room temperature), 440 ml of the supernatant was disposed of by suction and 600 μl of HCl (32%, wt/vol) was added to adjust the suspension to pH 2. The resulting suspension of silica coarse (SC) is sufficient for approximately 1,500 NA purifications. Small portions (4 ml) were made in glass bottles, tightly closed, and autoclaved for 20 min at 121°C to destroy any contaminating NA. SC is stable for at least 6 months when stored at room temperature in the dark.

(ii) Preparation of diatom suspension. The diatom suspension was made by adding 50 ml of H_2O and 500 μl of 32% (wt/vol) HCl to 10 g of high-purity, analytical grade Celite obtained from Janssen Chimica (Beerse, Belgium). The suspension was divided into aliquots into small glass bottles which were then tightly closed and autoclaved.

(iii) Buffers. Lysis buffer L6 was made by dissolving 120 g of GuSCN (Fluka Chemie AG, Buchs, Switzerland; catalog no. 50990) in 100 ml of 0.1 M Tris hydrochloride, pH 6.4 (Boehringer GmbH, Mannheim, Federal Republic of Germany); subsequently, 22 ml of a 0.2 M EDTA solution (Titriplex; Merck, Darmstadt, Federal Republic of Germany) adjusted with NaOH to pH 8.0 and 2.6 g of Triton X-100 (Packard Instrument Co., Inc., Downers Grove, Ill.) were added, and the solution was homogenized. Washing buffer L2 was made by dissolving 120 g of GuSCN in 100 ml of 0.1 M Tris hydrochloride, pH 6.4. Dissolution of GuSCN was facilitated by heating in a 60 to 65°C water bath under continuous shaking. Buffers L6 and L2 are stable for at least 3 weeks at room temperature in the dark. TE buffer (elution buffer) was 10 mM Tris hydrochloride–1 mM EDTA (pH 8.0), which was made from an autoclaved (20 min at 121°C) 100× concentrated stock solution.

Note of caution. Upon contact with acids, GuSCN can produce a toxic gas (HCN). As a precaution, GuSCN-containing buffers were prepared in a fume hood. As another precaution, GuSCN-containing waste was collected in a strong alkaline solution (10 N NaOH, in such an amount that the final concentration could not drop below 0.3 N).

Reaction vessels. Reaction vessels were prepared by adding 900 μl of lysis buffer L6 and either 40 μl of SC (protocol Y/SC) or 40 μl of diatom suspension (protocol Y/D) to a 1.5-ml Eppendorf reaction tube (Eppendorf type 3810) and homogenizing the solution by vortexing. In the experiments described here, the reaction vessels were assembled on the day of the extraction procedure. Results of other experiments (data not shown) have suggested that preassembled reaction vessels can be successfully used up to 1 week after assembly.

DNA and RNA purification by protocol Y/SC from serum and urine. First, the preassembled reaction vessel was vortexed to homogeneity, a 50-μl sample of serum or urine was then added, and the vessel was immediately vortexed (approximately 5 s). After 10 min at room temperature, the vessel was vortexed again (5 s) and centrifuged (15 s) in an Eppendorf microfuge (fixed angle, 12,000 × *g*), and the supernatant was disposed of by suction. The silica-NA pellet was subsequently washed (see below) twice with washing buffer L2, twice with ethanol 70% (vol/vol), and once with acetone. After disposal of the acetone, the vessels were dried at 56°C with open lids in an Eppendorf (Hamburg, Federal Republic of Germany) heat block for 10 min. Elution buffer (in the experiments described here, TE buffer with or without an RNase inhibitor; see technical remarks below) was added, and the vessel was closed, vortexed briefly, and incubated for 10 min at 56°C. The vessel was briefly vortexed again and centrifuged for 2 min at 12,000 × *g*, and the supernatant containing DNA and RNA was used for further experiments.

DNA and RNA purification by protocol Y/D from cell-rich sources. This protocol is essentially the same as protocol Y/SC, except that it uses diatoms rather than size-fractionated SC and allows for the purification of relatively large (10 to 20 μg) amounts of genomic NA as shown for gram-negative bacteria. Bacteria (5 to 10 μl) were scraped from plates and suspended in the test tube, which was further processed as described above for protocol Y/SC. Alternatively, 1 ml of an overnight culture of *Escherichia coli* K-12 HB101 containing a high-copy plasmid was centrifuged (30 s at 12,000 × *g*), and the bacterial pellet was suspended in 50 μl of TE buffer. This suspension was used as input material and processed as described above for protocol Y/SC.

Washing procedure. Silica-NA or diatom-NA pellets were washed by the addition of 1 ml of the appropriate washing solution and then vortexed until the pellet was (visually) completely resuspended. After centrifugation (15 s at approximately 12,000 × *g*), the supernatant was disposed of by suction (see technical remarks below).

NA. Bacterial plasmid DNA was purified from *E. coli* HB101 as described by Ish-Horowicz and Burke (12) followed by column chromatography with Sepharose CL2B (Pharmacia, Inc., Uppsala, Sweden) and ethanol precipitation. pCMV-E contains a 0.4-kilobase (kb) human cytomegalovirus DNA fragment cloned in the 2-kb vector pHC624 (3). In those cases in which *E. coli* carrying pCMV-E was mixed with human serum or urine specimens, the pellet of 150 μl of an overnight culture was mixed with 50 μl of these specimens. pEBV-10 contains a 0.9-kb Epstein-Barr virus DNA fragment cloned in the same vector. To obtain a plasmid preparation enriched for relaxed circular (CII) molecules, pEBV-10 DNA (2.9 kb) was treated with DNase I. HMW, MMW, and LMW DNA represent high-molecular-weight DNA (phage lambda DNA; 48 kb, 250 ng/μl; Boehringer GmbH), medium-molecular-weight DNA (*Hin*dIII digest of HMW DNA; fragment lengths of 23, 9.4, 6.7, 4.4, 2.3, 2.0, and 0.56 kb), and low-molecular-weight DNA (*Hpa*II digest of pHC624; fragment lengths of 471, 404, 242 [two fragments], 190, 147, 110, and 67 base pairs [bp] and some additional smaller fragments of undetermined lengths), respectively. After endonuclease treatment, MMW and LMW DNA were purified by one phenol-chloroform-isoamylalcohol (24/24/1, vol/vol/vol) and one chloroform-isoamyl alcohol extraction (24:1, vol/vol) and then ethanol precipitated. The precipitate was washed once with 70% ethanol (vol/vol), dried, and finally dissolved in TE buffer to reach DNA concentrations of 500 μg/ml (MMW DNA) or 2,000 μg/ml (LMW DNA) as determined photospectrometrically. Single-stranded phage M13 DNA (0.25 μg/μl) and *E. coli* 23S and 16S rRNA (2 μg/μl) were from Boehringer GmbH.

DNA labeling. Nicked pEBV-10 DNA (see above) was labeled with [α-^{32}P]dCTP (Amersham International, Amersham, United Kingdom) by the random priming method (Boehringer GmbH) to a specific activity of 7×10^7 cpm/μg. Unincorporated nucleotides were removed by Sepharose

CL2B (Pharmacia) column chromatography. The labeled product migrated on gels in the 1.8- to 2.4-kb range. HMW DNA was labeled by nick translation (20) to a specific activity of 10^7 cpm/μg; unincorporated nucleotides were removed by Sepharose CL2B chromatography. The labeled product migrated in agarose gels in the 48-kb range.

Enzymes. Restriction enzymes, RNase A, DNase I, and T4 DNA ligase were from Boehringer GmbH and were used as recommended by Maniatis et al. (15). Restriction enzyme digestions and ligase reactions were performed in 25- to 35-μl reaction volumes with 10 and 2 U, respectively, for 1 h at 37°C.

Clinical specimens. Frozen human urine or frozen human serum samples were randomly chosen from a bank; NA or bacteria were mixed with these samples after they had reached room temperature.

Bacterial strains. Bacterial strains were kindly provided by the World Health Organization Collaborative Centre for Bacterial Meningitis, Department of Bacteriology, University of Amsterdam, Amsterdam, The Netherlands.

Gel electrophoresis. In all experiments, NA was electrophoresed (8 to 10 V/cm) through neutral agarose slab gels containing ethidium bromide (1 μg/ml) in the buffer system (40 mM Tris–20 mM sodium acetate–2 mM sodium EDTA adjusted to pH 7.7 with acetic acid; ethidium bromide was added to a concentration of 1 μg/ml of buffer) described by Aaij and Borst (1). In those cases in which detection of rRNA was the major aim, we used gels prepared from previously autoclaved (20 min at 121°C) agarose solutions; gels were electrophoresed at 4°C, not submerged into the electrophoresis buffer to minimize RNA degradation. Photographs (Kodak Tri-X pan) were taken by UV transillumination (Shandon, Runcorn, United Kingdom) of the gel.

Technical remarks. (i) Preparation of GuSCN-containing buffers. In those cases in which NA is purified for PCR purposes, GuSCN-containing buffers (L6 and L2) may be purified from contaminating NA by filtration over glass columns with a diatom matrix (e.g., Celite, Janssen Biochimica, Leuven, Belgium). Dry columns and other glassware can be used after pretreatment for 5 h at 500°C. All experiments described in this paper were performed with nonfiltered buffers; results of several experiments have indicated that similar data can be obtained by using buffers filtered as described above.

(ii) Washing procedure. It is essential that the silica-NA or diatom-NA pellets are completely suspended in the washing solutions. Especially in the first wash, pellets may occasionally be very tight (which is directly dependent on the amount of HMW DNA), and it may take up to 1 min to suspend such pellets by vortexing; in the next washes, suspension can then be achieved without difficulty. It appears that some commercially available vortex machines are not powerful enough for suspension in these cases. In the experiments described here, we used a Monomixer (Sarstedt, AG, Rommelsdorf, Nümbrecht, Federal Republic of Germany). For the sedimentation of diatom-NA complexes, we used a 24-hole Hettich KG centrifuge (Tutlingen, Federal Republic of Germany) which was run until 10,000 rpm was reached (approximately 15 s).

(iii) Elution. In most rRNA purification experiments described here, elution was in TE buffer with an RNase inhibitor (either RNAsin [Promega Biotec] or vanadyl-ribonucleoside complexes [Sigma] at 0.5 U/μl and 20 mM, respectively). Results of several experiments have suggested that the presence of these inhibitors in the elution buffer is not strictly necessary for the recovery of undegraded rRNA (see Fig. 8C). When eluted NA is to be used for subsequent enzymatic treatment, care should be taken to recover the supernatant free of silica particles, since these may interfere with enzymatic reactions.

RESULTS

Initial experiments showed that silica particles, glass particles, or diatoms could be successfully used for the purification of NA from relatively pure NA-containing specimens (like restriction enzyme digests or agarose slices) in the presence of highly concentrated solutions of GuSCN. Since it was a major goal to evaluate our protocols with respect to reproducibility when tested on relatively impure and highly variable clinical specimens like serum and urine, we will mainly present here a representative selection of experiments obtained for such specimens.

Since NA is usually present in serum or urine in amounts too low to be visualized by UV illumination of ethidium bromide-NA complexes, exogenous NA was added to these specimens in the form of purified DNA (purified by classical methods; see Materials and Methods) or bacterial cells (*E. coli*) carrying a high-copy plasmid. To evaluate the purification of LMW DNA, a plasmid digest (fragment range, 471 to less than 67 bp) was used; for MMW DNA, a *Hin*dIII digest

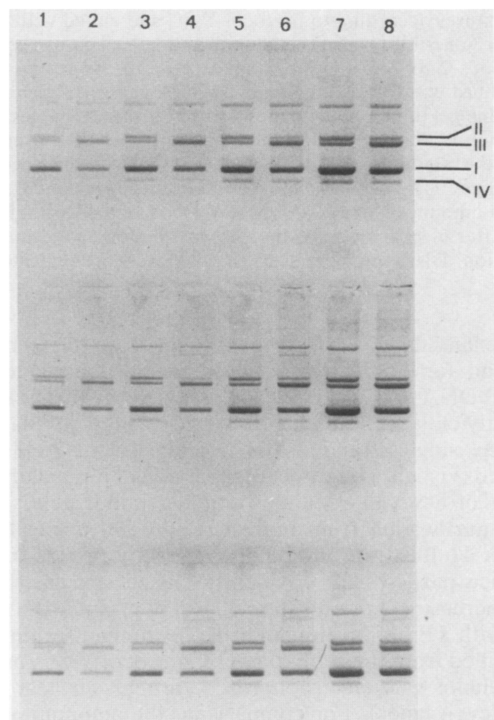

FIG. 1. Covalently closed circular DNA-binding capacity. Increasing amounts of pHC624 (5, 10, 20, and 40 μg for lanes 2, 4, 6, and 8, respectively; in a constant volume of 16 μl of TE buffer) were mixed with 50 μl of TE buffer (top panel), human urine (middle panel), or human serum (lower panel). DNA was extracted from these mixtures according to protocol Y/SC and eluted with 200 μl of TE buffer. Twenty-microliter samples were electrophoresed through a 0.8% agarose gel and photographed under UV illumination. Marker lanes 1, 3, 5, and 7 contain the amounts of DNA that would be observed at 100% recovery (500 ng, 1 μg, 2 μg, and 4 μg, respectively). The covalently closed (I), relaxed circular (II), and linear (III) forms as well as the alkaline supercoil (IV) of pHC624 are indicated.

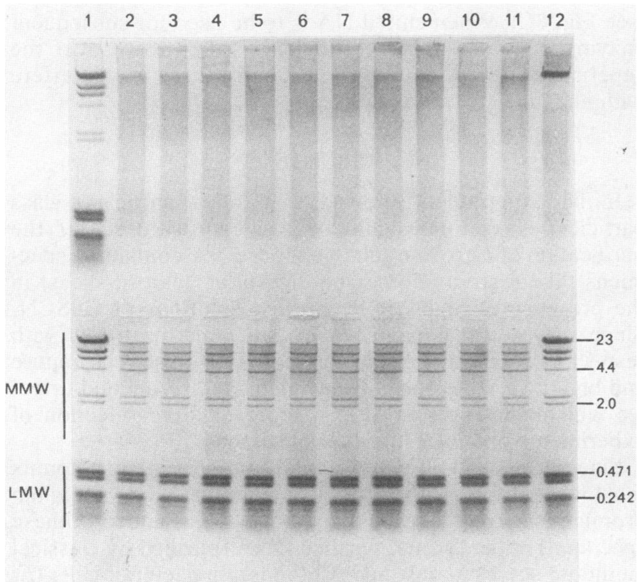

FIG. 2. Purification of DNA from human urine specimens. Known amounts of LMW DNA (6 μg) and MMW DNA (2 μg) were mixed with 50-μl human urine samples (numbered 1 to 10; samples 8 and 10 were clear, samples 1, 4, 5, 7, and 9 were turbid, and samples 2, 3, and 6 were very turbid). DNA was extracted from these mixtures according to protocol Y/SC and eluted with 75 μl of TE buffer. One-third was directly applied to a 1% agarose gel (lower panel, lanes 2 to 11 for urine samples 1 to 10, respectively), and another third was first treated with T4 DNA ligase and then applied to the same gel (upper panel, lanes 2 to 11 for urine samples 1 to 10, respectively). Marker lanes 1 and marker lane 12 (lower panel) contain the amounts of LMW and MMW DNA that would be observed at 100% recovery. Marker lane 12 (upper panel) contains the same amount of LMW and MMW DNA treated with T4 DNA ligase. After electrophoresis, the gel was photographed under UV illumination. DNA fragment sizes (in kilobases) are indicated.

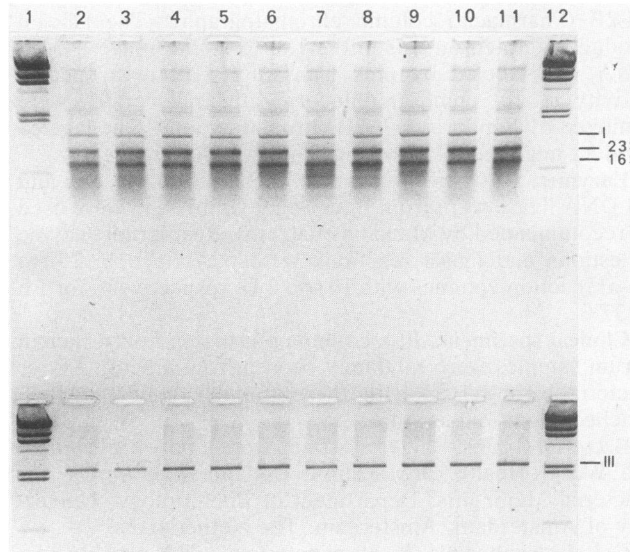

FIG. 3. Purification of DNA and rRNA from human urine. Ten different human urine specimens (numbered 1 to 10; samples 4, 5, 6, and 7 were clear, samples 1, 2, 3, and 8 were turbid, and samples 9 and 10 were very turbid) were mixed with *E. coli* carrying a 2.4-kb plasmid. NA was purified according to protocol Y/SC and eluted in 75 μl of TE buffer with RNAsin (0.5 U/μl). One-third of the eluate was directly applied to a 1% agarose gel (top panel, lanes 2 to 11), and another third was digested with *Eco*RI in the presence of RNase A (40 ng/μl) and applied to the same gel (lower panel, lanes 2 to 11 for urine samples 1 to 10, respectively). Marker lanes 1 and 12 contain 1 μg of MMW DNA. Form I (representing the uncut plasmid), form III (the 2.4-kb linearized plasmid), and bacterial rRNAs (23S and 16S) are indicated.

of phage lambda DNA (fragment range, 23 kb to 560 bp) was used; and for HMW DNA, 48-kb phage lambda double-stranded DNA was used. We also studied the purification of covalently closed circular (form I) and relaxed circular (form II) DNA, single-stranded DNA, and rRNA. Yields were estimated (visually) by direct comparison of input and output amounts of DNA in ethidium bromide-stained gels.

DNA purification from human serum and urine: binding capacity. To illustrate the binding capacity of size-fractionated silica particles for covalently closed circular (form I) DNA, increasing amounts (5 to 40 μg) of plasmid DNA were mixed with TE buffer, human urine, or human serum. DNA was purified from these mixtures by protocol Y/SC, and 1/10 of the eluate was electrophoresed through an agarose gel (Fig. 1, even lanes). For comparison, the amounts of DNA which would be observed at 100% recovery were also loaded on the same gel (Fig. 1, odd lanes). The data show that recovery of form I molecules exceeded 50%; no significant differences were observed for TE buffer, human serum, or human urine. In this experiment, the binding capacity for form I molecules exceeded 20 μg; results of other experiments (not shown) have suggested that the binding capacity exceeds 100 μg. Note that part of the form I or form II (relaxed circular) molecules have been converted into the linear form (form III), which is presumably the result of shearing occurring during the washing procedure.

DNA purification from human urine: recovery of linear double-stranded DNA and DNA ligase susceptibility. To illustrate the purification of linear double-stranded DNA in the range from 23 kb to less than 60 bp, 10 randomly chosen human urine specimens were mixed with a constant amount of MMW and LMW DNA. DNA was purified from these mixtures by protocol Y/SC, and one-third of the eluate was directly applied to the gel (Fig. 2, lower panel, lanes 2 to 11). Another third was treated with T4 DNA ligase and then applied to the same gel (Fig. 2, upper panel). The data show that no significant differences were observed for these urine specimens with regard to overall DNA recovery or DNA ligase susceptibility. DNA ligase treatment resulted in an overall increase in molecular weight; on the original photograph, the apparent smear was resolved into numerous discrete DNA fragments. For DNA fragments of less than 4 kb, yields were estimated to be at least 50%. The apparent lower recovery of intact fragments over 4 kb in size is presumably due to shearing, resulting in a background smear (see also Fig. 6B).

Simultaneous purification of DNA and rRNA from human urine. Another 10 human urine samples were mixed with *E. coli* carrying a 2.4-kb plasmid, and NA was subsequently purified from these mixtures by protocol Y/SC and eluted in TE buffer with an RNase inhibitor. One-third of the eluate was directly applied to the gel (Fig. 3, upper panel, lanes 2 to 11), and another third was restricted with *Eco*RI (which linearizes the plasmid) in the presence of RNase A (Fig. 3, lower panel, lanes 2 to 11).

The data suggest that rRNA can be purified from *E. coli* present in human urine in an almost undegraded form and that copurified plasmid DNA is sufficiently pure to be

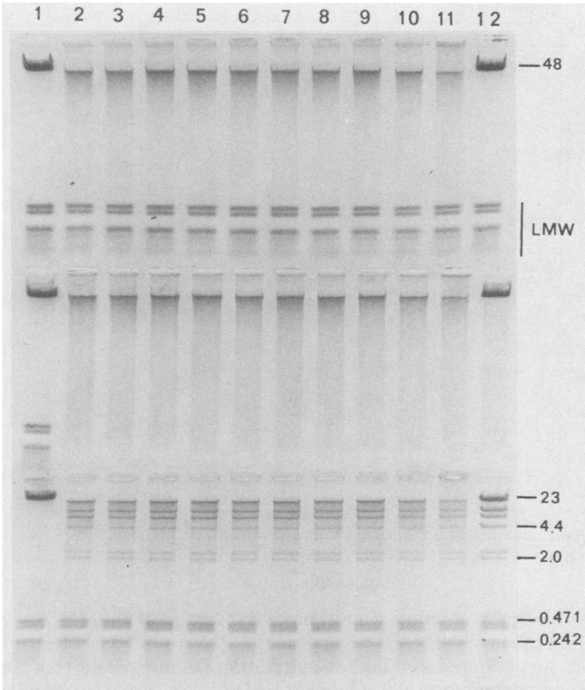

FIG. 4. DNA purification from human serum. Fifty-microliter samples of 10 different human serum specimens (numbered 1 to 10) were mixed with LMW and HMW DNA (8 μg and 4 μg, respectively, in a 16-μl volume). DNA was purified from these mixtures according to protocol Y/SC and eluted with 80 μl of TE buffer. One-fourth of the eluate was directly applied to the gel (top panel, lanes 2 to 11 for samples 1 to 10, respectively), one-fourth was treated with T4 DNA ligase (middle panel), and one-fourth was cleaved with *Hin*dIII (lower panel) and then applied to the same gel (1% agarose). Marker lanes 1 and marker lane 12 (top panel) contain the amount of DNA that would be observed at 100% extraction efficiency. Marker lanes 12 contain the same amount of DNA as that in lanes 1, either T4 ligase treated (middle panel) or *Hin*dIII digested (lower panel). DNA fragment sizes (in kilobases) are indicated.

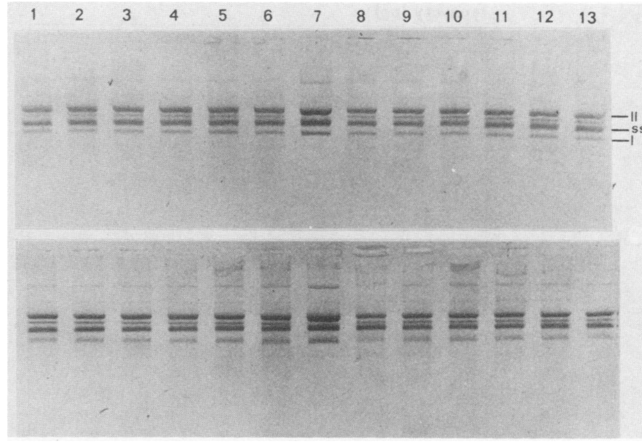

FIG. 5. Recovery of covalently closed, relaxed circular, and single-stranded DNA from human serum. Fifty-microliter samples of 24 randomly chosen human serum specimens were mixed with known amounts of DNA (a mixture of 2 μg of CII-rich pEBV-10 and 500 ng of phage M13 single-stranded DNA). DNA was purified according to protocol Y/SC and eluted with 50 μl of TE buffer. Half of the eluate was electrophoresed through a 1% agarose gel and photographed under UV illumination. Marker lanes 7 contain the amount of DNA that would be observed at 100% recovery. Lanes 1 to 6 and 8 to 13 in the top panel represent serum specimens 1 to 6 and 7 to 12, respectively; the corresponding lanes in the lower panel represent serum specimens 12 to 24. Single-stranded (ss) DNA, form I, and form II plasmid DNA are indicated.

completely cleaved by endonuclease treatment. The nature of the rRNA bands was confirmed by comigration with 16S and 23S rRNA preparations from commercial sources.

Purification of DNA from human serum: enzyme susceptibility. Ten human serum specimens randomly chosen from a bank were mixed with HMW DNA (48 kb) and LMW DNA. DNA was extracted from these mixtures by protocol Y/SC, and part of the eluate was electrophoresed through an agarose gel (Fig. 4, top panel, lanes 2 to 11). Another part was treated with T4 DNA ligase (Fig. 4, middle panel, lanes 2 to 11) or was cleaved with *Hin*dIII (Fig. 4, lower panel, lanes 2 to 11) and then applied to the same gel. These data show that no significant differences were observed for the different serum specimens. LMW DNA was recovered with high yields (over 50%) and was a good substrate for DNA ligase. HMW DNA purified from these serum specimens was a good substrate for *Hin*dIII and was recovered with an estimated yield of approximately 50% (Fig. 4, lower panel). The apparent lower recovery of the intact 48-kb molecule (top panel) is presumably due to shearing. Results of other experiments with radiolabeled HMW DNA were consistent with this interpretation (see also Fig. 6B).

Recovery of relaxed circular and single-stranded DNA from human serum. Since it is one of our future aims to detect hepatitis B virus in serum by PCR, we tested the purification procedure with regard to relaxed circular double-stranded DNA (form II) and single-stranded DNA, as a model for the hepatitis B virus DNA genome which is present in virions as a partially double-stranded, partially single-stranded relaxed 3.2-kb circular DNA (17).

Twenty-four human serum specimens were mixed with a 2.9-kb plasmid preparation (enriched for form II molecules) together with single-stranded DNA. DNA was purified from these mixtures by protocol Y/SC and electrophoresed (Fig. 5). Yields for form II and single-stranded DNA were estimated to be 50%; no major variations were observed for these 24 serum specimens. For five of these serum specimens, short nucleosomal ladders could be observed on the original negative; the occurrence in serum of such chromatin breakdown products at relatively low concentrations (up to 4 μg/ml of serum in some systemic lupus erythematosus patients) is well known (2, 19).

Nanogram and picogram recovery. To test the recovery of picogram amounts of DNA, ^{32}P-labeled DNA was mixed with a human serum, extracted according to protocol Y/SC, and counted in a liquid scintillation counter. The data presented in Fig. 6A suggest that, at the picogram level, DNA was recovered with a yield of approximately 40%. Addition of carrier DNA (500 ng) to the serum samples increased the yield to approximately 60%. When picogram recovery from the 24 serum specimens mentioned above was tested in the same way (6 pg of ^{32}P-labeled DNA together with 500 ng of carrier DNA), yields varied from 40 to 53%, with a mean of 48 ± 4% (standard deviation).

Some of the data presented (Fig. 4, top panel) suggest a relatively low rate of recovery of HMW DNA. However, when low amounts (100 pg to 100 ng) of ^{32}P-labeled HMW DNA were mixed with human serum and extracted by protocol Y/SC, yields were approximately 50% over a broad DNA concentration range (Fig. 6B).

Recovery of intact rRNA from human serum. RNases

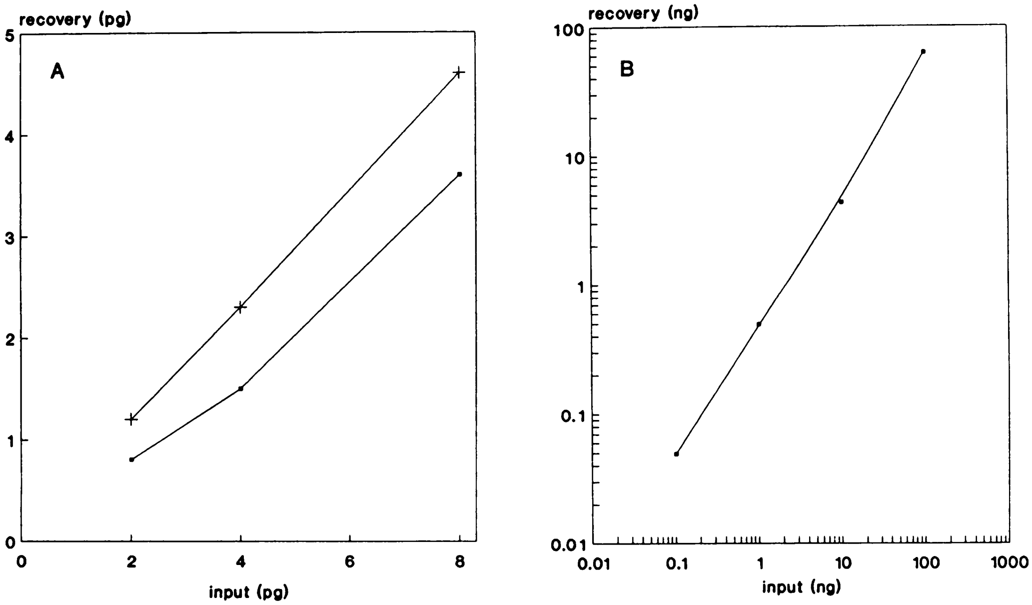

FIG. 6. Nanogram and picogram recovery of DNA from human serum. (A) Picogram amounts (input) of ^{32}P-labeled pEBV-10 DNA were mixed with 50-μl samples of a human serum with (+) or without (■) the addition of carrier DNA (500 ng of HMW DNA per 50 μl of serum). DNA was extracted according to protocol Y/SC, and half (50 μl) of the eluate was counted in a liquid scintillation counter. Data represent the means of four separate extractions after correction for background. (B) Nanogram amounts of ^{32}P-labeled HMW DNA (input) were mixed with 50 μl of human serum. DNA was extracted according to protocol Y/SC, and half (100 μl) of the eluate was counted in a liquid scintillation counter (recovery). Data represent the mean for three separate extractions after correction for background.

present in serum rapidly degrade unprotected (naked) RNA such as previously purified rRNAs. This is illustrated in Fig. 7 (top panel), which shows 10 randomly chosen human serum specimens which were mixed with 23S and 16S rRNAs and processed according to protocol Y/SC within seconds after the addition of the rRNAs. When purified rRNAs were instead added to the serum lysate, rRNA was recovered apparently undegraded and with high (over 60%) yields, suggesting that rRNA degradation did not take place during the purification procedure (results not shown). When the same 10 serum specimens were mixed with *E. coli* (or mammalian cells; not shown) in which rRNA is protected from degradation by serum nucleases, rRNAs were recovered with similar efficiencies and almost undegraded (Fig. 7, lower panel). Notice that the relatively low recovery of bacterial genomic DNA (see Discussion) is somewhat exaggerated in this figure because we routinely electrophorese neutral RNA gels not submerged into the electrophoresis buffer. In such cases, the presence of high amounts of rRNA precludes staining of the slower-migrating genomic DNA.

Use of diatoms for the purification of NA from cell-rich sources. In human serum or urine, only low amounts of genomic NA are usually present, which allows for the use of size-fractionated silica particles (SC) for efficient NA purification from these sources, as suggested above. However, when SC was used for the purification of relatively large amounts (over 5 μg) of genomic (bacterial or mammalian cellular) DNA, the purification procedure had to be stopped because of the formation of very tight SC-NA complexes which could no longer be redispersed (washed). This problem was solved by simply substituting SC for diatoms (protocol Y/D). Diatoms are the fossilized cell walls of unicellular algae and consist almost entirely of silica (27) but are much larger than SC particles, possibly allowing for greater shearing forces to disrupt diatom-NA networks by

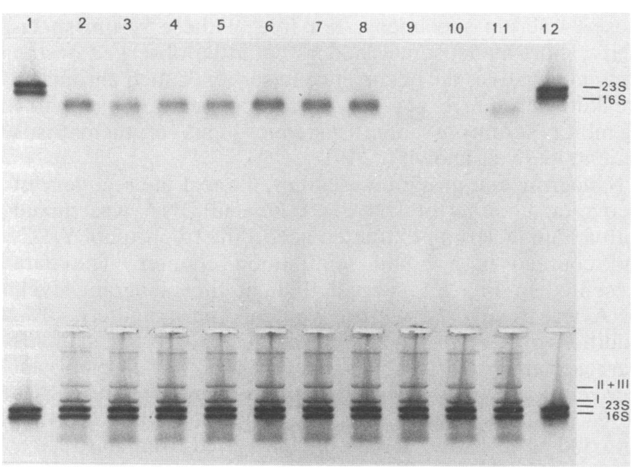

FIG. 7. Recovery of intact rRNAs from human serum. (Top) Fifty-microliter samples of 10 different human serum specimens were mixed with 16S and 23S rRNAs (4 μg), immediately processed according to protocol Y/SC, and eluted in 60 μl of TE buffer with RNAsin. Half of the eluate was applied to a 0.8% agarose gel (lanes 2 to 11 represent serum samples 1 to 10, respectively). Marker lanes 1 and 12 contain the amount of rRNA that would be observed at 100% recovery. (Bottom) Fifty-microliter samples of the same 10 human serum specimens were mixed with *E. coli* carrying a 2.4-kb plasmid, and NA was extracted from these mixtures by protocol Y/SC and eluted with 40 μl of TE buffer with RNAsin. Half of the eluate was electrophoresed in the same gel (lanes 2 to 11 represent serum samples 1 to 10, respectively). Marker lanes 1 and 12 contain 2 μg of *E. coli* rRNA. Forms I, II, and III plasmid DNA and 23S and 16S rRNAs are indicated.

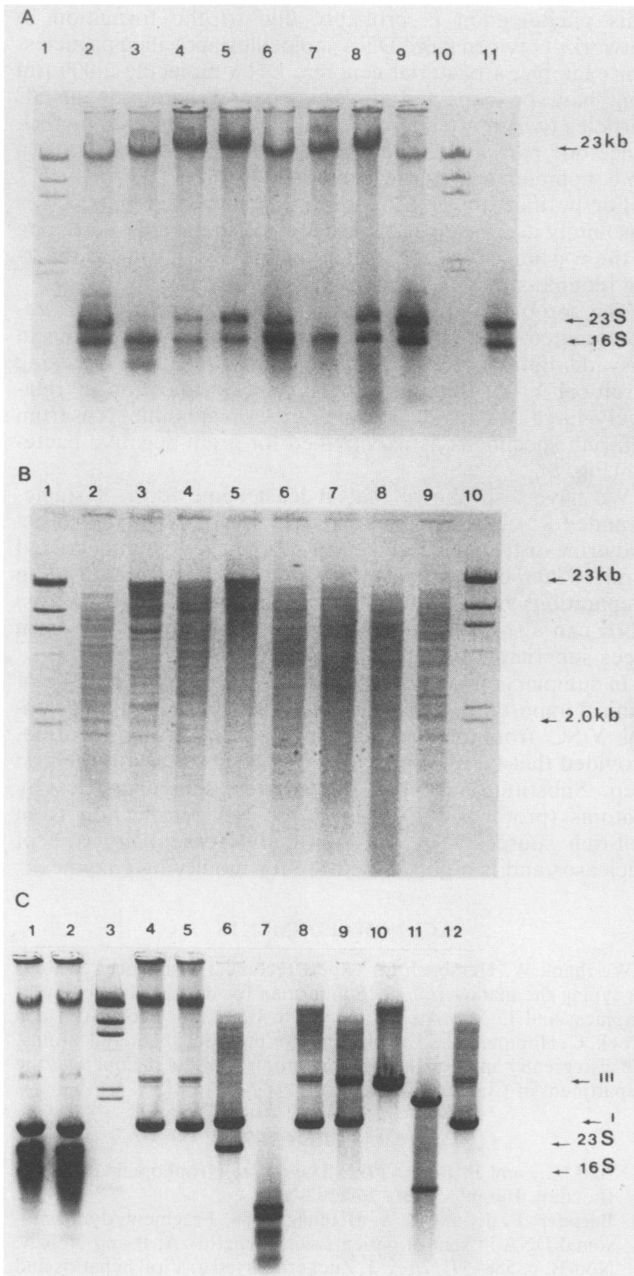

FIG. 8. Recovery of NA from cell-rich sources (protocol Y/D). (A) Bacteria (approximately 5 to 10 μl) were scraped from plates, and NA was purified according to protocol Y/D. Elution of NA was with 60 μl of TE buffer with 20 mM vanadyl-ribonucleoside complexes, and 20 μl was electrophoresed through a 0.8% agarose gel. Lanes 1 and 10, 1 μg of phage lambda DNA digested with *Hin*dIII; lane 11, 2 μg of *E. coli* rRNA; lane 2, *N. meningitidis*, serogroup B, serotype 4 (lab no. 891237); lane 3, *H. influenzae* type b (lab no. 891163); lane 4, *K. pneumoniae* (lab no. 880102); lane 5, *E. coli* (lab no. 890968); lane 6, *Y. enterocolitica* serotype O9; lane 7, *S. typhimurium*; lane 8, *P. aeruginosa* (lab no. 891167); lane 9, *N. gonorrhoeae* (lab no. 830563). (B) The same procedure as that described for panel A was performed, except that NA was eluted in 60 μl of TE buffer and 20 μl was simultaneously treated with RNase A (15 ng) and 10 U of a restriction enzyme for 1 h at 37°C. Electrophoresis was through a 0.8% agarose gel. Lane 1 and 10, 1 μg of phage lambda DNA digested with *Hin*dIII; lane 2, *N. meningitidis* DNA digested with *Dra*I; lane 3, *H. influenzae* DNA digested with *Pvu*II; lane 4, *K. pneumoniae* DNA digested with *Nco*I; lane 5, *E. coli* DNA digested with *Sph*I; lane 6, *Y. enterocolitica* DNA digested with *Dra*I; lane 7, *S. typhimurium* DNA digested with *Dra*I; lane 8, *P. aeruginosa* DNA digested with *Sph*I; lane 9, *N. gonorrhoeae* DNA digested with *Dra*I. (C) A 1-ml portion of an overnight culture of *E. coli* HB101 carrying a high-copy-number plasmid (pCMV-E) was centrifuged, and the bacterial pellet was suspended in 50 μl of TE buffer. NA was purified from this suspension according to protocol Y/D, NA was eluted with 50 μl of TE buffer (without RNase inhibitors), and 20 μl was used for gel electrophoresis through a 1% agarose gel. Lanes 1 and 2, NA without treatment; lane 3, 2 μg of lambda DNA digested with *Hin*dIII; lanes 4 and 5, NA after RNase digestion; lanes 6 to 12, NA after a 1-h digestion with 15 U of *Bgl*I, *Cfo*I, *Cla*I, *Eco*RV, *Eco*RI, *Hin*fI, and *Pvu*II (in the presence of RNase A), respectively. The plasmid contains a single recognition site for *Eco*RI and no recognition sites for *Cla*I, *Eco*RV, and *Pvu*II. 23S and 16S rRNAs, form I (covalently closed), and form III (linear) plasmid DNA are indicated. Photographs were made by UV transillumination of the gels.

vortexing (see Discussion). Figure 8A shows the NA purification results for eight different pathogenic gram-negative bacterial species, scraped from plates and processed according to protocol Y/D. The average molecular weight of the recovered genomic DNA was estimated to be at least 30 kb. In a similar experiment (in which NA was eluted without vanadyl-ribonucleoside complexes which, at 20 mM, inhibit restriction enzymes), purified genomic DNAs of the same bacterial species appeared to be good substrates for several restriction enzymes (Fig. 8B); the amount of genomic DNA should be sufficient for at least 100 Southern blot analyses for the detection of single-copy genes with labeled probes.

We make most of our DNA recombinants in the high-copy vector pHC624, developed by Boros et al. (3), which consistently gives yields between 10 and 20 mg of plasmid DNA per liter of an overnight culture, at least in *E. coli* K-12 HB101, when purified by classical methods (see Materials and Methods). Figure 1C shows the results for NA purifications by protocol Y/D for *E. coli* HB101 carrying such a high-copy-number recombinant; the amount of NA in each lane corresponds to 400 μl of an overnight culture (see Materials and Methods). Both plasmid DNA and genomic DNA appeared to be good substrates for restriction enzymes (Fig. 8C, lanes 4 to 12).

Figure 8C (lanes 1 and 2) also suggests that rRNAs were recovered essentially undegraded, as might be expected from the data for serum and urine specimens presented above. In this particular experiment, elution was in TE buffer without RNase inhibitors like RNAsin or vanadyl-ribosyl complexes. Results of several other experiments (not shown) have shown that undegraded rRNAs can be purified by protocol Y without the use of such inhibitors. The differences in rRNA patterns as observed in Fig. 8A (e.g., lanes 2 and 3) for different bacterial species are apparently highly reproducible, indicating diagnostic or taxonomic significance (C. J. A. Sol et al., manuscript in preparation).

DISCUSSION

In this paper, we have described a method (protocol Y/SC) for the rapid purification of NA from human serum or urine. In these clinical materials, NA may be present in mammalian or procaryotic cells as well as in viruses. Whether NA can be purified directly (i.e., without pretreatment) from these sources by protocol Y/SC will depend on the nature of the particular organism. Most mammalian cells and viruses are expected (6, 10, 11, 26, 28) to lyse in the first step of the procedure, releasing their NA which can then bind to the silica particles and subsequently be purified. Whereas this method has been suitable for purification of NA from several pathogenic gram-negative bacterial species like *Neisseria meningitidis*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *E. coli*, *Yersinia enterocolitica*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Neisseria gonorrhoeae* (Fig. 8), we have, until now, not been able to purify NA directly from gram-positive bacteria (e.g., *Staphylococcus aureus* and *Streptococcus pneumoniae*) or yeasts (e.g., *Candida albicans* and *Saccharomyces cerevisiae*), presumably because of poor lysis.

A major aim has been to develop a reliable, rapid, and simple method for routine NA purification from clinical specimens like serum or urine, one that might be less prone to sample-to-sample transmission of NA during purification (in view of extremely sensitive assays for NA detection like PCR). Evaluation of NA transmission during purification and evaluation of subpicogram recovery of NA by PCR, however, await the redesign of our laboratory. Preliminary experiments have suggested that human immunodeficiency virus RNA (T. Wolfs, personal communication) and hepatitis B virus DNA can be detected by PCR following purification from patient serum specimens by protocol Y/SC.

We have shown that by protocol Y/SC, DNA (single-stranded, double-stranded, covalently closed, or relaxed circular) is recovered from serum or urine with high yields (usually over 50%) and is a good substrate for DNA-modifying enzymes like restriction enzymes or DNA ligase. Results of other experiments (not shown here) have suggested that other enzymes like reverse transcriptase, *E. coli* DNA polymerase, or *Taq* (*Thermus aquaticus*) DNA polymerase can also be successfully used.

Double-stranded DNA in the size range of 48 kb to approximately 60 bp was recovered with efficiencies of approximately 50%, although significant shearing occurred for the larger DNA molecules (Fig. 2, 4, and 6B). At present, we have no indications that DNA recovery is dependent on its nucleotide sequence (Fig. 2, lower panel). Only very small DNA fragments (less than approximately 40 to 60 bp) have apparently been lost during purification (not shown). Results of experiments with radiolabeled DNA suggested that picogram amounts were recovered with similar efficiencies; addition of carrier DNA to the samples resulted in a slight but significant increase in yields (Fig. 6A). These results also suggested that DNA yields were almost independent of the amount of DNA present in the starting material, from the picogram to the nanogram level (Fig. 6B).

When radiolabeled HMW DNA (approximately 48 kb) was followed throughout the purification procedure from serum, approximately 8% of the DNA was apparently not bound to the silica particles, 8% was lost during the washing procedures (mainly in the second ethanol wash [5%]), and approximately 30% of the DNA was not eluted. These figures were the same for DNA input amounts ranging from 100 pg to 100 ng (not shown).

The DNA-binding capacity for CI molecules of the size-fractionated silica particles is high (over 20 μg/40 μl of SC) (Fig. 1) and will usually far exceed the amounts present in small specimens of serum or urine.

When such relatively large amounts of genomic (bacterial or mammalian) DNA were used as input material for protocol Y with size-fractionated SC as NA carrier, the SC-NA complexes were so tight that they could no longer be redispersed (washed) by vortexing and the purification procedure had to be stopped (see technical remarks above). This phenomenon is probably due to the formation of networks between large DNA molecules and silica particles. For example, a bacterial genomic DNA molecule 2,000 μm long may be expected to bind several hundreds of SC particles (which are approximately 2 to 10 μm in diameter). Since one particle will bind to several DNA molecules at a time, complex aggregates will arise.

For purification of NA present in such aggregates, it is absolutely necessary that these are redispersed by vortexing in the washing solutions (which, inevitably, results in shearing [double-stranded breakage] of the DNA).

This problem was solved by simply substituting size-fractionated SC for the much larger diatoms, resulting in easy disruption of NA-diatom complexes upon vortexing (protocol Y/D), thus allowing for the purification of relatively large (10 to 20 μg) amounts of genomic NA from cell-rich specimens as exemplified for gram-negative bacteria (Fig. 8).

We have also shown that at least some form of single-stranded RNA (rRNA) is copurified with DNA from serum and urine or from bacterial cultures in an almost undegraded form. Other experiments (R. Boom et al., manuscript in preparation) have shown that double-stranded (rotavirus) RNA can also be successfully purified by protocol Y from feces supernatants.

In summary, it seems that most NA types that may be of clinical importance can be simultaneously purified by protocol Y/SC from clinical specimens like serum or urine, provided that lysis of the target organism occurs in the first step. Substitution of size-fractionated silica particles by diatoms (protocol Y/D) allows for NA purification from cell-rich sources. NA thus purified is essentially free of nucleases and is a good substrate for modifying enzymes.

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